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Patentanmeldung Nr. Patent application No. Demande de brevet n°

03291677.7 ✓

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R C van Dijk



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INSTITUT NATIONAL DE LA RECHERCHE
AGRONOMIQUE
145, Rue de l'Université
75007 Paris
FRANCE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
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If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Method of producing double low restorer lines of Brassica napus having a good
agronomic value

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The invention relates to a method of producing a double low restorer lines of *Brassica napus* for Ogura cytoplasmic male sterility (cms) presenting a radish introgression carrying the Rfo restorer genes deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from *Brassica oleracea*, and having a good 5 agronomic value characterized by female fertility, a good transmission rate of Rfo and a high vegetative vigour. The invention relates also to a method of forming *Brassica napus* hybrid seed and progeny thereof and to the use of markers for selection.

Breeding restorer lines for the Ogu-INRA Cytoplasmic Male Sterility (cms) system 10 in rapeseed (*Brassica napus* L.) has been a major objective during the past few years. Extensive backcross and pedigree breeding were necessary to improve their female fertility and to get double low restorer lines. The so-called « double low » varieties are those low in erucic acid in the oil and low in glucosinolates in the solid meal remaining after oil extraction. However some difficulties can still be 15 encountered in breeding these lines (introgression rearrangements, possible linkage with negative traits) due to the large size of the radish introgression.

The inventors thus assigned themselves the objective of providing a new improved double low restorer line with a good agronomic value.

This objective is obtained by a new method of producing a recombined double low 20 restorer line for the Ogu-INRA cms in rapeseed.

A first object of the present invention relates to a method of producing double low restorer lines of *Brassica napus* for Ogura cytoplasmic male sterility (cms) presenting radish introgression carrying the Rfo restorer gene deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from *Brassica oleracea*, and having 25 a good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour, said method including the step of:

a) crossing double low cms lines of spring *Brassica napus* comprising a deleted radish insertion with the double low line of spring Drakkar for forming heterozygous restored plants of *Brassica napus*,

30 b) irradiating before meiosis the heterozygous restored plants obtained in step a) with gamma ray irradiation,

- c) crossing pollen from flowers obtained in step b) with the cms double low spring Wesroona line,
- d) testing the progeny for vigour, female fertility and transmission rate of the cms gene,

5 5 e) selecting progeny lines.

A method according to claim 1, wherein the irradiation dose in step b) is 65 Gray during 6 mn.

According to one advantageous form of embodiment of the method according to the present invention, the double low cms line of spring *Brassica napus* of step a) is

10 10 R211.

R211 is an INRA spring restorer line.

Drakkar is a French spring registered variety.

Wesroona is an Australian spring registered variety.

According to one advantageous form of embodiment of the method according to the

15 15 present invention, the testing is performed with the combination of five markers selected from PGIol, PGIUNT, PGInt, BolJon and CP418.

Another object of the present invention relates to double low restorer lines of *Brassica napus* for Ogura cms presenting a Rfo insertion deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from *Brassica oleracea*, and having a

20 20 good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour.

According to one advantageous form of embodiment, the double low restorer lines present a unique combination of five markers selected from PGIol, PGIUNT, PGInt, BolJon and CP418.

25 25 Another object of the present invention relates to a method of forming *Brassica napus* hybrid plants and progeny thereof obtained through the steps of:

- a) providing a restorer line produced according to claim 1 and bred to be homozygous,
- b) using said restorer line in a hybrid production field as the pollinator,

30 30 c) using cms sterile plants in a hybrid production field as the hybrid seed producing plant, and

- d) harvesting the hybrid seed from the male sterile plant.

Another object of the present invention relates to seeds of Brassica plant obtained from the methods according to the present invention.

Still another object of the invention relates to seeds of Brassica napus deposited in NCIMB Limited, 23 St Machar Drive, Aberdeen, Scotland, AB24 3RY, UK, on

5 July 4, 2003, under the reference number NCIMB41183.

Another object of the present invention relates to the use of the combined markers PGiol, PGIUNT, PGIint and BolJon for selecting recombined restorer lines of Brassica napus for Ogura cms presenting a Rfo insertion deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a 10 good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour.

Such markers are represented in the following figures for the R2000 line.

According to one advantageous form of embodiment, the present invention relates to:

- 15 - The marker PGiol which is amplified using the primers: PGiol U and PGiol L
(PGiol U: 5'TCATTTGATTGTTGCGCCTG3';
PGiol L: 5'TGTACATCAGACCCGGTAGAAAA3')
- The marker PGIint which is amplified using the primers: PGIint U and PGIint L
(PGIint U: 5'CAGCACTAATCTTGCAGGTATG3';
PGIint L: 5'CAATAACCCTAAAAGCACCTG3')
- 20 - The marker PGIUNT which is amplified using the primers: PGiol U and PGIint L:
(PGiol U: 5'TCATTTGATTGTTGCGCCTG3';
PGIint L: 5'CAATAACCCTAAAAGCACCTG3')
- The marker BolJon which is amplified using the primers: BolJon U and BolJon L:
25 (BolJon U: 5'GATCCGATTCTTCTCCTGTTG3';
BolJon L: 5'GCCTACTCCTCAAATCACTCT3')
- The marker CP418 which is amplified using the primers: SG129 U and pCP418 L:
(SG129 U: cf Giancola et al, 2003 *Theor Appl. Genet. (in press)*
pCP418 L : 5'AATTCTCCATCACAAAGGACC3')
- 30 Another object of the present invention relates to the combination of five markers PGiol, PGIUNT, PGIint, BolJon and CP418 whose sequences are described in the appending figures.

In the annexed drawing that follows, the following abbreviations are used :

Dra	Drakkar
Rel-15-1, E38,R15	R2000
Hete, Hel	heterozygous R211*Drakkar
5 Darm	Darmor
Bol:	Brassica oleracea
Bra, B.rap:	Brassica rapa
GCPA18-A19, Wes, Aust:	Wesroona
Sam, Sam1PGIolSunt5	Samourai
10 RRH1, ba2c	RRH1
<ul style="list-style-type: none"> - <u>Figure 1</u> illustrates Gamma ray Irradiation and F2 production. - <u>Figure 2</u> illustrates seed set on 'R211' and 'R2000'. - <u>Figure 3</u> illustrates the number of seeds per pod of different lines. - <u>Figure 4</u> illustrates PGIol primer localisation on the segment of PGI sequence from 	
15 Data Base. In that figure:	
PGIol:	<ul style="list-style-type: none"> - primer PGIol U (named in SGAP: BnPGICh 1 U) - primer PGIol L (named in SGAP: Bn PGICh 1 L)
PGIint:	<ul style="list-style-type: none"> - primer PGIint U - primer PGIint L (is out side the sequence, after 500bp).
20	<ul style="list-style-type: none"> - <u>Figure 5</u> illustrates electrophoresis gel of PGI-2 gene (PGIol), PCR marker and SG91, a PCR marker close to Rfo. - <u>Figure 6</u> illustrates Pgi-2 segment of DNA amplified by PCR with PGIol primers. - <u>Figure 7</u> illustrates digestion of the PCR product PGIol by Mse1.
<p>In that figure:</p>	
25	<p>Sam and Darm has a 75bp band.</p> <p>Drak, R211.Dk and R2000 showed a 70pb one (Acrylamide 15%).</p> <p>8 was similar to Samourai (75bp) ; mix with Drakkar (70pb) it allowed the visualisation of the two bands.</p> <p>- <u>Figure 8</u> illustrates electrophoresis gel of PGIUNT marker.</p>
30	<p>In that figure:</p> <p>PGIUNT band (about 950bp) is present in B.oleracea, B.rapa cv Asko, maintainer and restored lines except in 'R211'.</p>

There is no amplification in radish and Arabidopsis.

In various Brassica genotypes only one band was amplified. Size band are similar but sequences are different.

Sequence of Restored lines RRH1 and R113 are homologous to B.rapa sequence.

5 Sequence of R2000 is homologous to Drakkar and B.oleracea sequences.

- Figure 9 illustrates electrophoresis gel of PGIint PCR marker.

In that figure PGIint of radish line 7 is of about 950bp. This band is the same as in the restored RRH1 and R113. It is not found in R211. It is not either in R2000. However the PGIint band is of a similar size in the various Brassica species.

10 - Figure 10 illustrates PGIint and PGIUNT of B.oleracea group sequences.

- Figure 11 illustrates PGIint and PGIUNT of B.rapa group sequences: the numbers indicates the base substitutions between the two sequences and del is for a deletion in B. oleracea sequence.

- Figure 12 illustrates comparison between B.olearecea and B.rapa PGI sequences.

15 - Figure 13 illustrates the comparison of Drakkar, Wesroona R2000, B.oleracea and R.rapa PGI sequence comparison.

- Figure 14 illustrates comparison of RRH1, R113, which is similar to B.rapa and different from B.oleracea.

- Figure 15 illustrates comparison of RRH1, R113 and Sam B.rapa on one side and

20 R2000, Drakkar and Wesroona and B.oleracea on the other side.

- Figure 16 illustrates Bo1Jon PCR marker.

- Figure 17 illustrates CP418 marker.

In that figure, the CP418 band (about 650bp) is specific to the B.oleracea genome. It is present in B.ol, B.napus (Samourai, Drakkar, Pactol and the herterozygous

25 R2111*Dk). It is absent from the restored rapeseed (RRH, R113 and R211). It is present in the homozygous R2000.

- Figure 18 illustrates summary markers table.

It should be understood, however, that the example is given solely by way of illustration of the object of the invention, of which they in no way constitute a

30 limitation.

Example I: method of producing a double low restorer line of *Brassica napus* for Ogura cytoplasmic male sterility (cms) presenting a radish introgression, carrying the Rfo restorer gene deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from *Brassica oleracea*, and having a good 5 agronomic value characterised by female fertility, a good transmission rate of Rfo) and a high vegetative vigour.

Materials and methods :

Genotypes: The 'R211' line with a deleted radish insertion was crossed to the spring low GLS rapeseed 'Drakkar' to produce a F1 progeny ('R211*Dk'). The spring low 10 GLS cms line 'Wesroona' (australian origin) was used for following crosses. Were used as control in molecular analyses: Winter restored lines derived from 'Samourai' carrying the complete ('RRH1') or incomplete ('R113') introgression as well as European radish line7, Asiatic restored radish D81, wild radish, *Brassica oleracea*, and *B.rapa* cv Asko, *Arabidopsis thaliana*.

15 Gamma ray irradiation: Whole flowering plants were treated with gamma rays from a Co60 source in a controlled area. Subletal dose fo 65 Gray was applied before meioses. The chosen dose was used for further two experiments on 35 plants.

Testcrosses and F2 production: Irradiated plants were transferred in an insectproof greenhouse after removing flower buds larger than 2 mm. The irradiated F1 progeny 20 was used to handpollinate the cms 'Wesroona' line. The restored derived F1' plants were allowed to produce F2 families harvested individually and precisely sown in a field assay along with non irradiated controls (Fig 1).

Phenotypic selection: Three visual criteria were scored (on a 1 to 5 scale) over 2 years in field assays, on 1200 F2 offsprings plus 44 controls (82 330 quoted plants): 25 1-Vegetative vigour,
2- Normality of the ratio of fertile /sterile plants in the F2 segregation, and
3- Female fertility (pod development and seed set).

Advanced selfed generations of the selected families were obtained either in field or greenhouse and produced homozygous lines (F4) for further analysis.

30 Isozyme analysis was performed as in (Delourme R. and Eber F. 1992. *Theor Appl Genet* 85: 222-228), marker development from (Fourmann M et al 2002. *Theor Appl. Genet.* 105:1196-1206.): PCR products are validated by sequencing.

Alignments were made using Blast Ncbi and Uk Crop Net Brassica DB and the Multialin software INRA Toulouse.

Method :

We choose one low GLS spring homozygous restorer line, 'R211', already

5 exhibiting deletions in the introgression (Delourme R. and Eber F. 1992. *Theor Appl Genet* 85: 222-228. Delourme R et al 1998. *Theor Appl Genet* 97: 129-134. Delourme R. et al 1999. *10th Int. Rapeseed Congress, Canberra.*). Several molecular markers are missing on either side of Rfo, such as spATCHIA (Fourmann M et al 2002. *Theor Appl. Genet.* 105:1196-1206), spSG91 (Giancola S et al 2003 *Theor Appl. Genet. (in press)*)). 'R211' lost the isozyme expression of the Pgi-2 allele of the radish gene but also the one of Pgi-2 allele of *B.oleracea* genome (1,2). Moreover, the homozygous 'R211' shows linked negative traits such as low vigour and very poor seed set. We hypothesised that these plant lack a rapeseed chromosomal segment. The fertile ratio in F2 progenies derived from this material

10 is lower than expected (64% instead of 75%). We initiated the program from this 'R211' line and tried to force recombination between the Rfo carrying introgression from this deleted line and the rapeseed homologous chromosome from a double low *B. napus* line.

15

Ionising irradiation is known to induce chromosomal rearrangements by double

20 strand breaks followed by aberrant rejoining of the ends. Gamma-ray irradiation was used on a heterozygous F1 derived from the 'R211' line to induce chromosome breaks, just before meiosis, aiming at a recombination of the deleted radish introgression in the rapeseed genome.

Results :

25 Very few families were at the best score for the three criteria out of 1200 F2 families tested.

Only one, 'R2000', proved to produce a normal ratio of fertile plants per selfed progeny with a stable recovery of good agronomic traits such as a good female fertility, with a normal seed set compared to 'R211' (Fig 2 and 3). This family was

30 obtained from a 6 mn irradiation treatment.

Glucosinolate analysis confirmed its low content.

In figure 2 (Seed set on 'R211' and 'R2000') R2000 showed normal inflorescences, with a normal looking architecture.

In figure 3 (Number of seeds per pod), we observe:

- on the best 'R2000' F4 families in self pollination (Selfings) and in testcrosses
- 5 - on 'Pactol' cms line on rapeseed and 'R211' controls.

Example II: selection of markers in the Pgi-2 gene

PGI isoenzyme analysis: 'R2000' progeny expressed the rapeseed Pgi-2 allele from *B. oleracea* genome, originally lost in 'R211'.

10 Three PCR markers were defined to characterise the R2000 family compared to the known restorer rapeseed RRH1 and R113.

1) PGIdl marker was developed from the BrassicaDB sequences to be specific to the Brassica genome. There is no amplification in radish nor in *Arabidopsis th.*, but only in Brassica, with one 278 bp band.

15 2) PGInt marker amplified a longer part of the Pgi-2 gene, allowing clear distinction between the various tested species *Brassica*, *Raphanus* and *Arabidopsis*. The species *B.rapa* and *B.oleracea* were not distinguished by the band size, but by their PGInt band sequence.

3) PGInt marker, a combination of the PGI ol U and PGI int L primers.

20 This marker had the specificity of the PGIdl marker but amplifying a longer part as for PGInt one.

II.1 PGIdl marker

With the PGIdl primers, the 'R211' parental line showed no amplification, while the spring tested lines showed a 278bp band. Its DNA sequence is homologous to 25 the PGI-2 sequences from the Crop Net UK DB in Brassica species and from previous work in our group (named SGAP sequences) (Localisation of the primers SG PGI chou, Fig 4).

It was ortholog of the clone MJB21-12, on the chromosome V, (34543bp) in *Arabidopsis* (NCBI DB).

30 PGIdl plus SG91 to set an Homozygocity test:

The combined use of two sets of primers in a mix PCR, PGIdl marking the Pgi-2 gene and SG91 (from S. Giancola et al, Giancola S et al 2003 *Theor Appl. Genet.*

(*in press*)), a very close marker to the Rfo gene, was set up to discriminate homozygous from heterozygous plant among the fertile plants segregating in F2 progenies derived from 'R211'.

One family R2000 showed no difference between homozygote and heterozygote

5 offsprings:

The Pgi-2 gene is present in the R2000 homozygote, which is not the case for the parental homozygous R211.

In figure 5 (PGIol and SG91 PCR markers):

SG91 is located on the radish introgression very close to Rfo

10 The homozygous 'R2000' family has recovered the PGIol band.

DNA sequence of the band confirmed the homology with the known Arabidopsis and Brassica Pgi-2 sequence. Control genotypes (Drakkar, Pactol, and, Samourai, Darmor) had the same pattern on the gel. Sequence of this common band allowed to confirm their high homology as they were quasi similar except one base 15 substitution.

The homozygous 'R2000' family has recovered the PGIol band having the characteristic of the Drakkar group of cultivars. It was distinct from those of the Samourai group, including the known restorers.

20 This amplified part of the Pgi-2 is very conserved and hardly any differences were shown among the various genotypes. A longer part of Pgi-2 gene was investigated.

II.2 PGIUNT and PGIint markers

Electrophoresis Patterns of PCR products :

PGIUNT marker: A second reverse primer, PGIint L, was designed further down the Pgi-2 sequence, to amplify as well conserved and as variable regions of the gene.

25 When used with the PGIol U primer, it amplifies a 950bp band only in Brassica genomes.

R211 didn't show any band, The homozygous 'R2000' showed the PGIUNT band as in the Drakkar parent.

In figure 8 (PGIUNT marker) :

30 PGIint marker amplified a longer part of the Pgi-2 gene, allowing clear distinction between the tested species. B.rapa and Boleracea were not distinguished by the band size, but by their PGIint sequence. All tested restored genotypes, but the

‘R211’ line, exhibited the European radish band and one Brassica band, homologous to the *B.rapa* one.

The homozygous ‘R2000’ didn’t show the radish PGInt band, as in the deleted ‘R211’ parental line, but showed one Brassica band, homologous to the *B. oleracea*

5 one.

Electrophoresis of PGInt marker is represented in figure 9.

Sequence analysis:

Comparison of the PGI sequences from the data bases.

A PGI segment of about 490bp is known.

10 Sequences of a segment of about 490bp from different genotypes (*B. oleracea*, *B. rapa*, *B. napus*) have been studied in our laboratory group and some sequences were given to Brassica Crop Net DB: EMAF25875 to 25788 by M.Fouramnn (4) These sequences are very conserved.

Comparison of the *B. rapa* et *B. oleracea* species PGI sequences:

15 Comparison between PGI sequences we have obtained from the tested genotypes of *B. oleracea* and *B. rapa* species, showed that they were distinct by 19 base substitutions. These substitutions allowed to distinguish PGInt sequences from the other tested genotypes of rapeseed, homologous to either *B.rapa* cv Asko (RRH1 and R113) or *B. oleracea* (Drakkar, R211*DK but also R2000).

20

Example III: selection of marker in a region close to Rfo

Markers surrounding the Rfo gene in the radish insertion were determined in order to facilitate the Rfo gene cloning (Desloires S et al 2003 *EMBO reports* 4, 6:588-594). One of these, the SG129 PCR marker was located very close to Rfo

25 (Giancola S et al 2003 *Theor Appl. Genet. (in press)*): it co-amplified distinct bands in *B. oleracea* and *B. rapa* genomes of *B. napus*, but the radish band was very difficult to see on a gel.

The target SG129 sequence was ortholog of a clone (AC011000, at the locus F16P17) in *Arabidopsis thaliana*. This clone overlapped an *Arabidopsis* adjacent 30 contig clone (AC07190).

From the Brassica Crop Net DB, we found one *B. oleracea* clone, (EMBH448336, 764bp) blasting with the beginning of the A011000, and a second *B. oleracea* clone

(EMBH53971), distant from about 300bp on the Arabidopsis map, that blasted with the end of ACO7190.

We designed a new PCR marker, BolJon, between the two *B.oleracea* clones. We verified that it allowed amplification of a specific PCR bands in the different genotypes compared here.

In figure 22 (electrophoresis gel of BolJon PCR products):

- In *Arabidopsis*, a BolJon 815bp band was amplified, homologue to the overlapping segment of the contigs.
- In *Brassicaceae* diploid species, BolJon marker showed distinct bands: one of 950bp in *B.oleracea* and one of 870bp in *B.rapa*. It showed that the two *B.oleracea* clones (EMBH53971 and EMBH448336) are in sequence continuity in *Brassica* genome as it is for the ortholog sequences in *Arabidopsis*.
- In *B.napus*, these two bands are co-amplified in the maintainer lines, Samourai or Drakkar.
- 15 - In radish line7, one BolJon band was amplified of about 630 bp long. The band of the restored radish cmsRd81 was slightly smaller.
- In all the restored rapeseed lines, one of the BolJon bands was of the same size as the radish line7. BolJon is a marker of the radish introgression.
- The homozygous restored rapeseed lines, 'RRH1', 'R113' and also 'R211', 20 only showed the *B.rapa* band and the 630bp radish band bp suggesting the *B.oleracea* ortholog of the target gene is absent or has been modified when the radish segment of chromosome was inserted into the rapeseed *B.oleracea* constitutive genome.

'R2000' homozygote plants showed radish PCR BolJon, plus the two *Brassica* BolJon bands, again having recovered the *B.oleracea* one, lost in 'R211' and other restorer lines.

We designed a primer, pCP418L, specific of the *B.oleracea* genome in the tested species. With the SG129U primer it amplified only one PCR band (650bp) in the *B.oleracea* species. (Fig 23)

30 There was no amplification in *B.rapa*, in radish, nor in *Arabidopsis*, but there was a clear CP418 band in *B. napus* maintainer lines. Its sequence was strictly homologous to the EMBH448336 sequence. This marker was in a very conserved

DNA sequence allowing no polymorphism between genotypes except by presence / absence.

In RRH1, R113 and in R211 there was no CP418 band, indicating as previously that the Boleracea ortholog of the target gene is absent or has been modified following 5 the radish insertion.

‘R2000’ homozygote plants showed CP418 band, again having recovered the specific Boleracea one.

In the present invention, a new recombined low GLS restorer line has been selected with a good female fertility. The poor value of line ‘R211’ allowed selection in the 10 field for a rare recombination event and characterisation the ‘R2000’ family.

The homozygous ‘R2000’ presents a unique combination of the PGiol, PGIUNT, PGlint and BolJon markers when compared with the rapeseed restorer analysed yet: PGlinT marker showed that the homozygous restored rapeseed lines, RRH1 and R113 presented the European radish band plus one Brassica band, homologous to 15 B.rapa genome. ‘R2000’ shows no radish band, lost as in its parental deleted line R211, but showed one Brassica band homologous to Boleracea. The ortholog PGlinT sequence in its B.rapa genome is not amplified with this marker in R211 and Drakkar genetic background.

PGiol marker and PGIUNT marker sequences in restored lines RRH1 and R113 20 were homologous to the B.rapa cv Asko one. In ‘R2000’, PGIUNT sequence is homologous to Boleracea. The ortholog PGlinT sequence in its B.rapa genome is not amplified with this marker in R211 and Drakkar genetic background.

BolJon marker showed that the homozygous restored rapeseed lines, including ‘R211’ presented the European radish band plus only the B.rapa one. ‘R2000’ 25 shows the two bands of ‘R211’ plus the recovered Boleracea BolJon band.

CP418 marker showed that ‘R2000’ recovered this conserved Boleracea segment.

Our hypothesis is that a recombination event took place in the pollen mother cell which gave rise to ‘R2000’ plants. The deleted radish introgression was then integrated to the normal homologous chromosome segment, carrying the Boleracea 30 type Pgi-2 gene and BolJon target sequence, characterised by these markers, probably from the Drakkar ‘00’ genome present in the irradiated heterozygous ‘R211*DK’.

The pattern observed for BolJon suggests that the recombination event resulted in a particular duplicated region, one from radish and one *B. oleracea*, in the 'R2000' family.



CLAIMS

1. A method of producing double low restorer lines of *Brassica napus* for Ogura cytoplasmic male sterility (cms) presenting radis introgression carrying the Rfo restorer gene deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from *Brassica oleracea*, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour, said method including the step of:
 - 5 a) crossing double low cms lines of spring *Brassica napus* comprising a deleted radish insertion with the double low line of spring Drakkar for forming heterozygous restored plants of *Brassica napus*,
 - 10 b) irradiating before meiosis the heterozygous restored plants obtained in step a) with gamma ray irradiation,
 - c) crossing pollen from flowers obtained in step b) with the cms double low spring Wesroona line,
 - 15 d) testing the progeny for vigour, female fertility and transmission rate of the cms gene,
 - e) selecting progeny lines.
- 20 2. A method according to claim 1, wherein the irradiation dose in step b) is 65 Gray during 6 mn.
- 3 3. A method according to claim 1 wherein the double low cms line of spring *Brassica napus* of step a) is R211.
- 25 4. A method according to claim 1 wherein the testing is performed with the combination of five markers selected from PGIol, PGIUNT, PGIInt, BolJon and CP418.
- 30 5. Double low restorer lines of *Brassica napus* for Ogura cytoplasmic male sterility (cms) presenting a Rfo insertion deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from *Brassica oleracea*, and having a

good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour.

6. Double low restorer lines of *Brassica napus* according to claim 5, wherein
5 they present a unique combination of five markers selected from PGIol,
PGIUNT, PGInt, BolJon and CP418.
7. *Brassica napus* hybrid plants and progeny thereof obtained through the steps
of:
10 a) providing a restorer line produced according to claim 1 and bred to be
homozygous,
b) using said restorer line in a hybrid production field as the pollinator,
c) using cms sterile plants in a hybrid production field as the hybrid seed
producing plant, and
15 d) harvesting the hybrid seed from the male sterile plant.
8. The seeds of *Brassica* plant developed from the *Brassica* line obtained in
claim 1.
20 9. The seeds of *Brassica napus* obtained in claim 7.
10. The seeds of *Brassica napus* obtained in claims 1 and 2 deposited in NCIMB
Limited, 23 St Machar Drive, Aberdeen, Scotland, AB24 3RY, UK, on
July 4 , 2003, under the reference number NCIMB41183.
25
11. Use of the combination of five markers PGIol, PGIUNT, PGInt, BolJon and
CP418 for selecting RECOMBINED restorer lines of *Brassica napus* for
Ogura cms presenting a Rfo insertion deleted of the radish Pgi-2 allele and
recombined with the Pgi-2 gene from *Brassica oleracea*, and having a good
30 agronomic value characterised by female fertility, a good transmission rate
of Rfo and a high vegetative vigour.

12. Use according to claim 11, wherein:

- The marker PGIol is amplified using the primers: PGIol U and PGIol L
(PGIol U: 5'TCATTGATTGTCGCCTG3';
PGIol L: 5'TGTACATCAGACCCGGTAGAAAA3')
- 5 - The marker PGIint is amplified using the primers: PGIint U and PGIint L
(PGIint U: 5'CAGCACTAATCTGCGGTATG3';
PGIint L: 5'CAATAACCCTAAAAGCACCTG3')
- The marker PGIUNT is amplified using the primers: PGIol U and PGIint L:
(PGIol U: 5'TCATTGATTGTCGCCTG3';
PGIint L: 5'CAATAACCCTAAAAGCACCTG3')
- 10 - The marker BolJon is amplified using the primers: BolJon U and BolJon L:
(BolJon U: 5'GATCCGATTCTTCTCCTGTTG3';
BolJon L: 5'GCCTACTCCTCAAATCACTCT3')
- The marker CP418 is amplified using the primers: SG129 U and pCP418 L:
(SG129 U: cf Giancola et al (5)
pCP418 L : 5'AATTCTCCATCACAAAGGACC3')

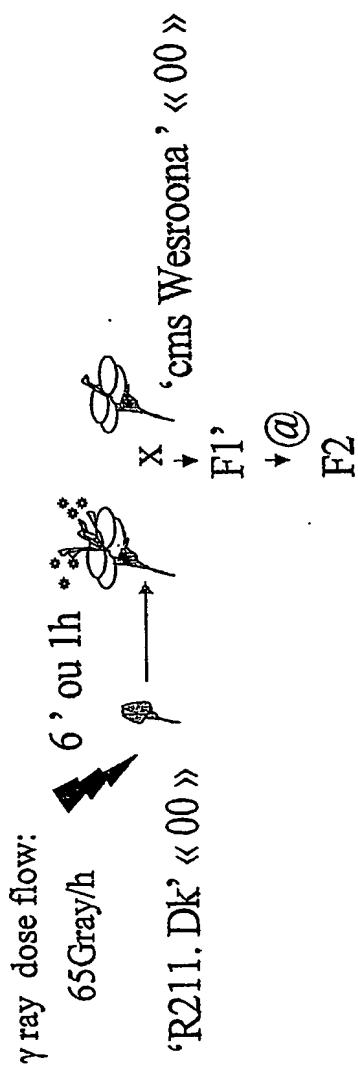
13. Combination of five markers PGIol, PGIUNT, PGIint, BolJon and CP418 whose sequences are described in the appending figures.

Method of producing double low restorer lines of *Brassica napus*
having a good agronomic value

ABSTRACT

A method of producing double low restorer line of *Brassica napus* for Ogura cytoplasmic male sterility (cms) presenting radish introgression carrying the Rfo restorer gene deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from *Brassica oleracea*, and having a good agronomic value characterized by female fertility, a good transmission rate of Rfo and a high vegetative vigour. A method of forming *Brassica napus* hybrid seeds and progeny thereof. The seeds of *Brassica napus* and use of the combined markers PG1ol, PG1unt, PG1int, BolJon and CP418 for selecting.

Fig 1



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Fig 2

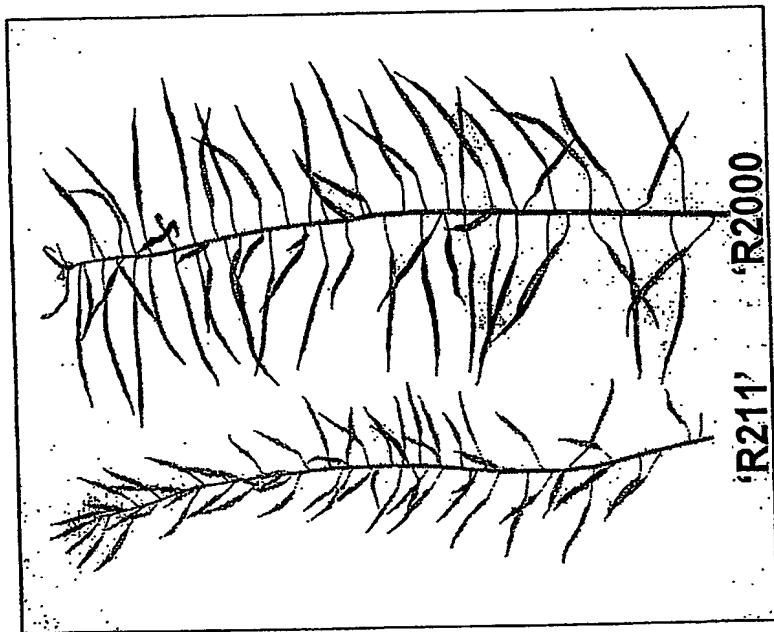


Fig 3

Genotype	Selfings	Test Crosses
Drakkar	29.3	
Pactol	23.1	
R211	11.2	25.5
R2000	26.5 (24.0 – 31.1)	27.0 (24.0 – 28.7)

Fig 4

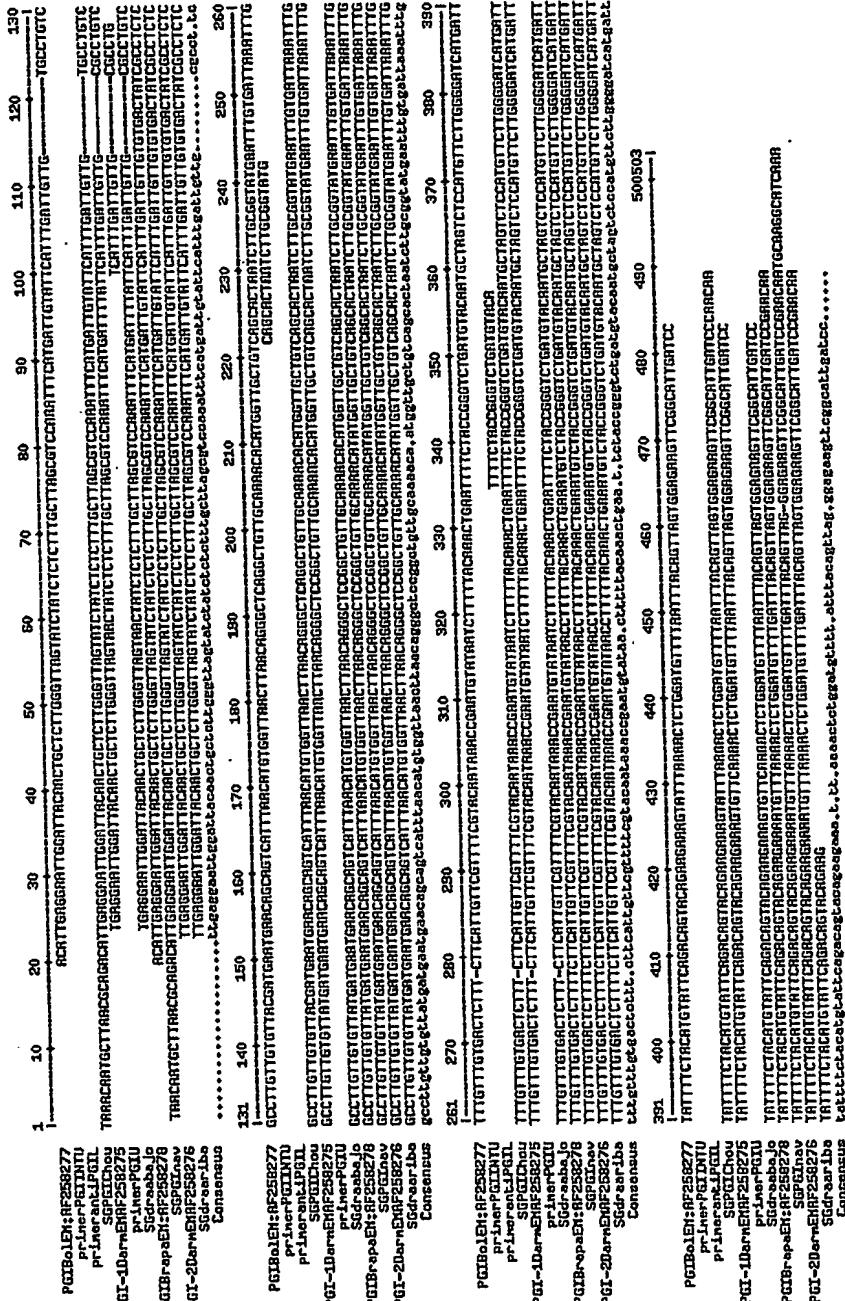


Fig 5

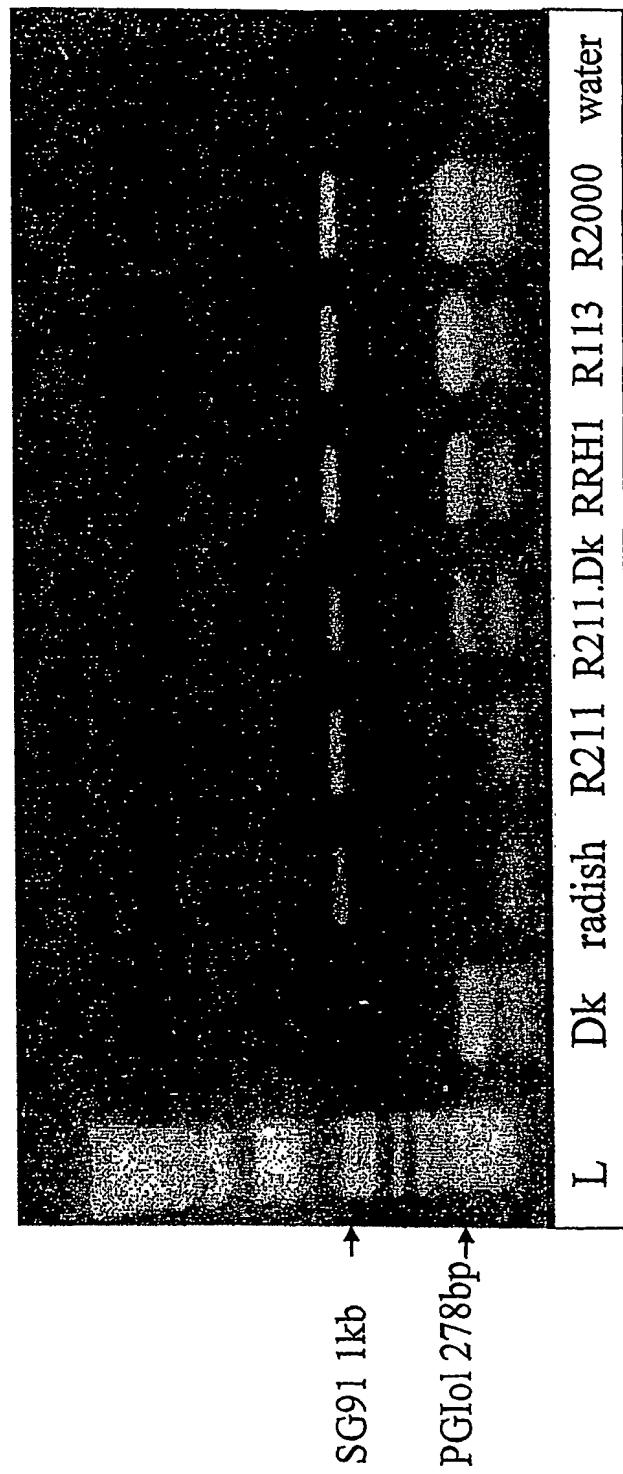
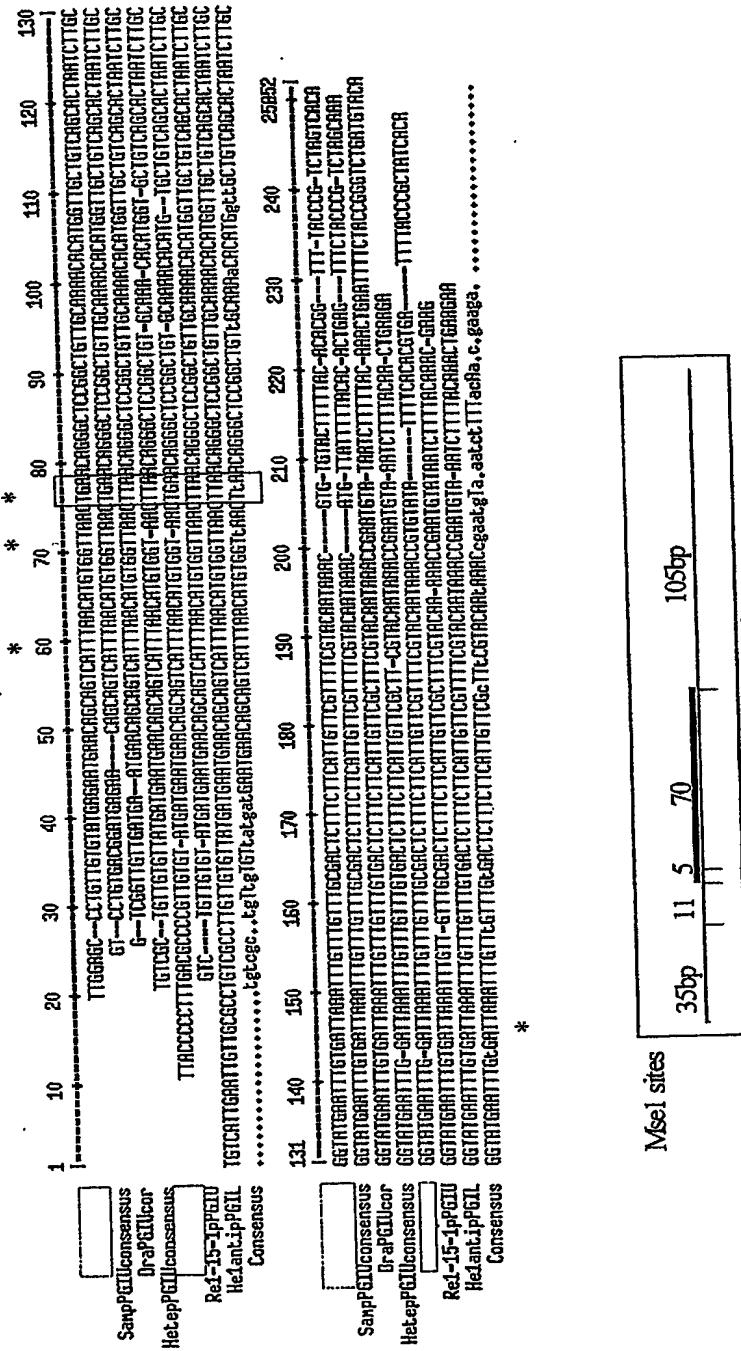
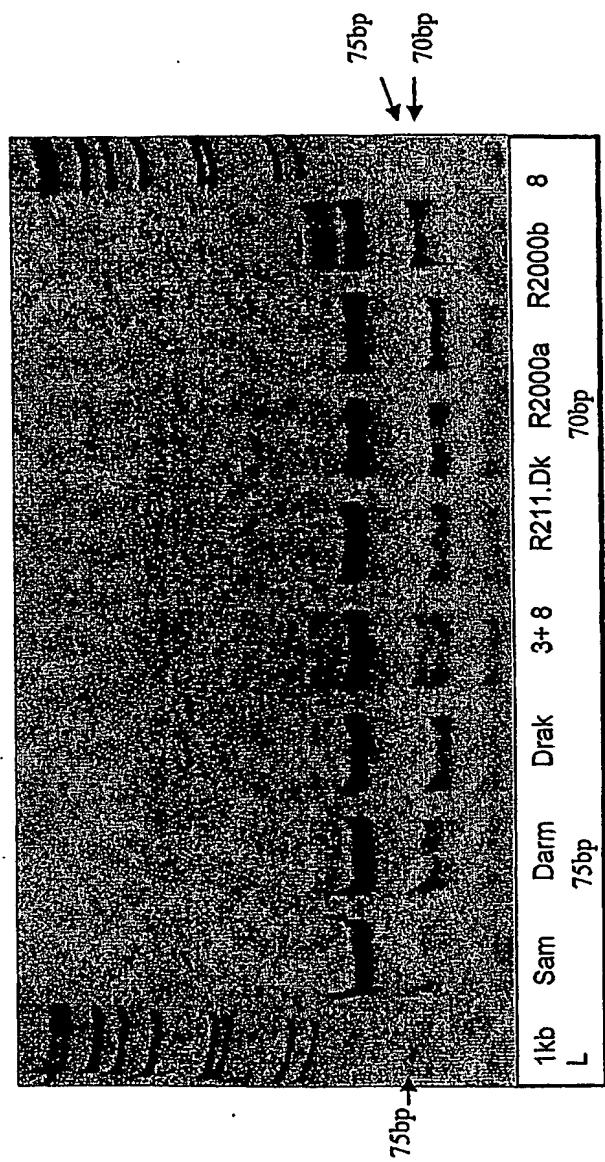


Fig 6

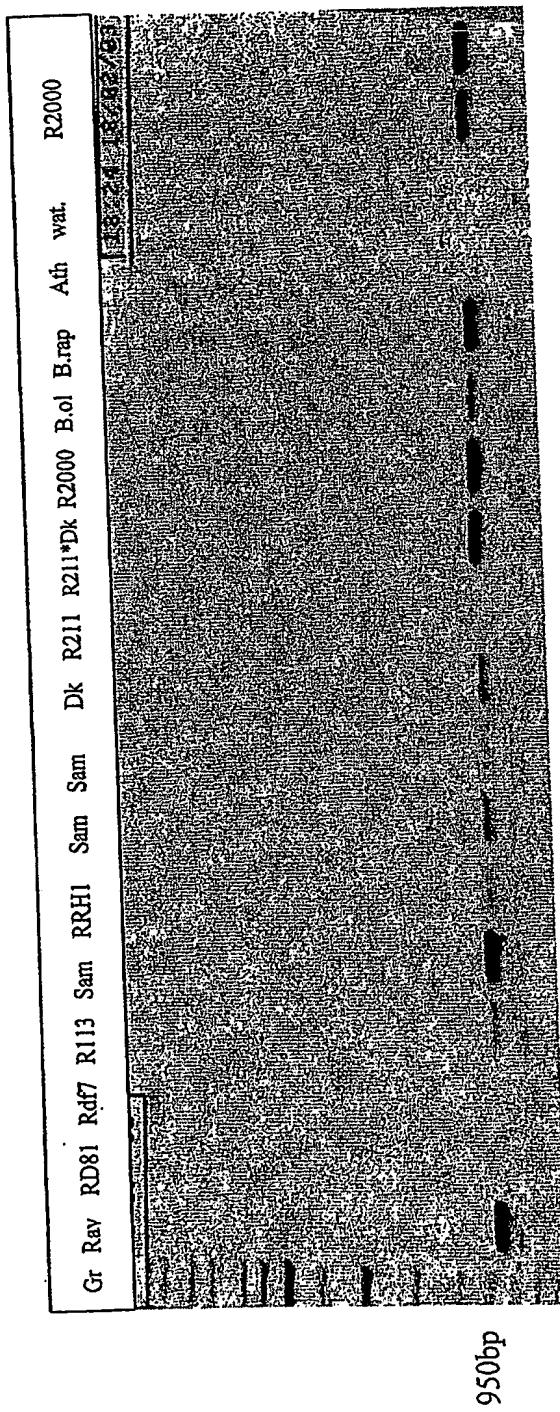


Mse1 restriction enzyme cut DNA sequences at the T/TAA sites (*)

Fig 7



8
Fig



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Fig. 9.

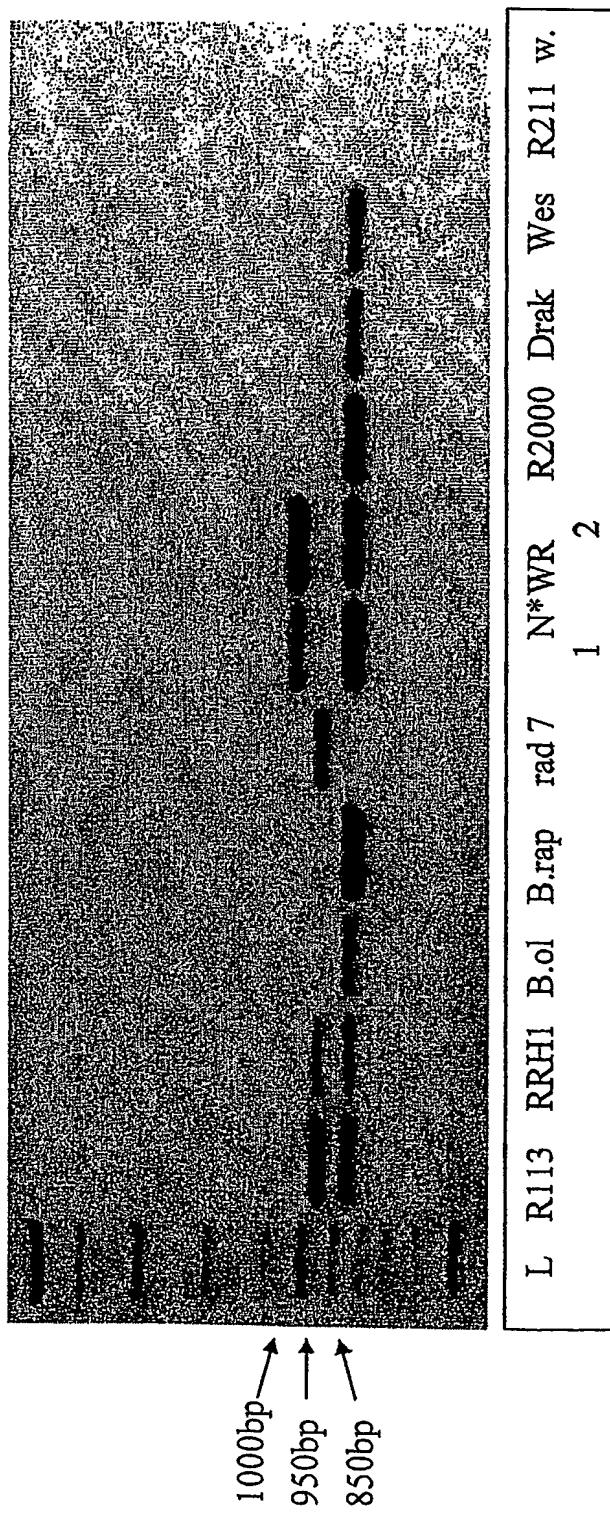


Fig 10

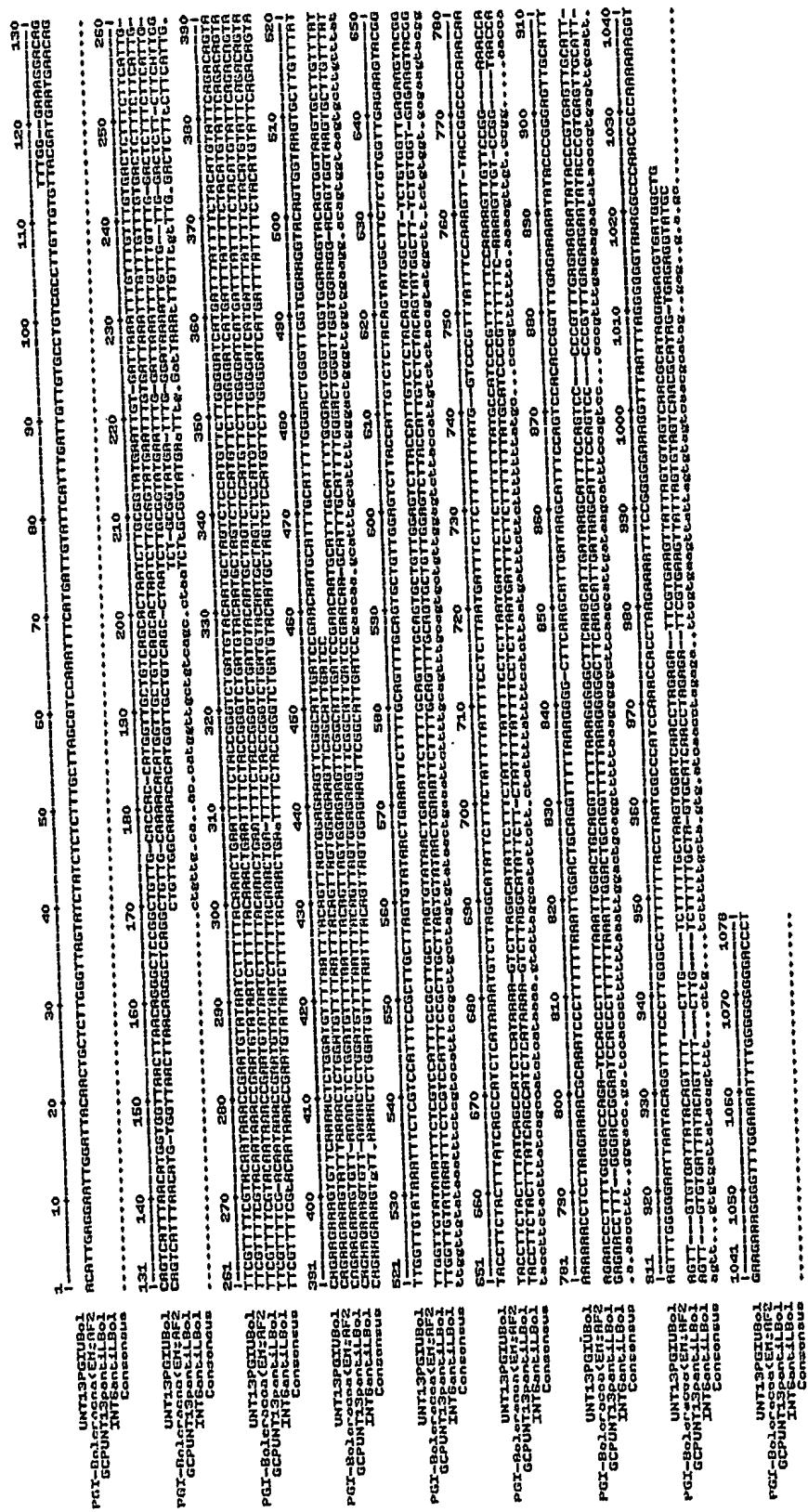


FIG 11

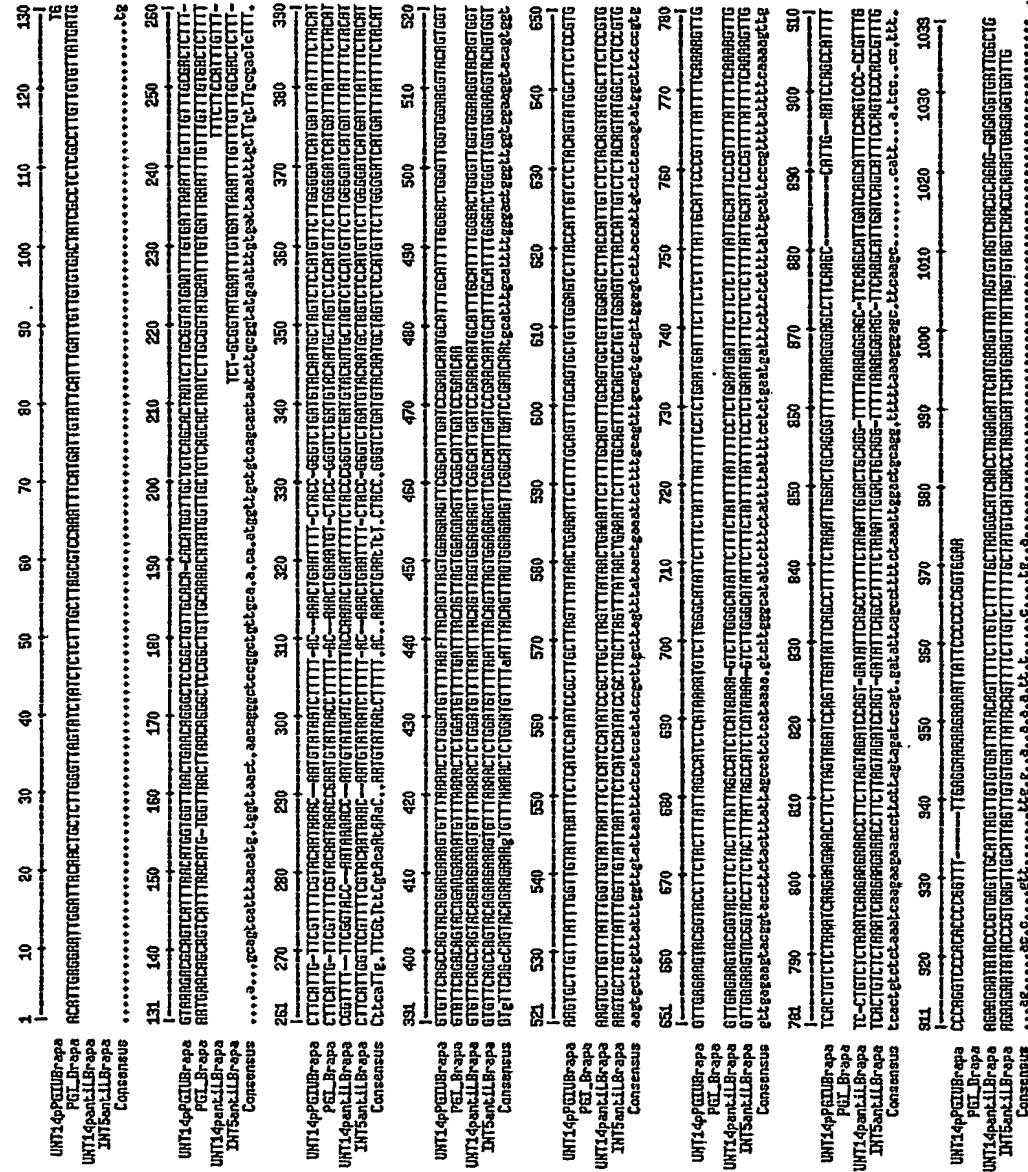
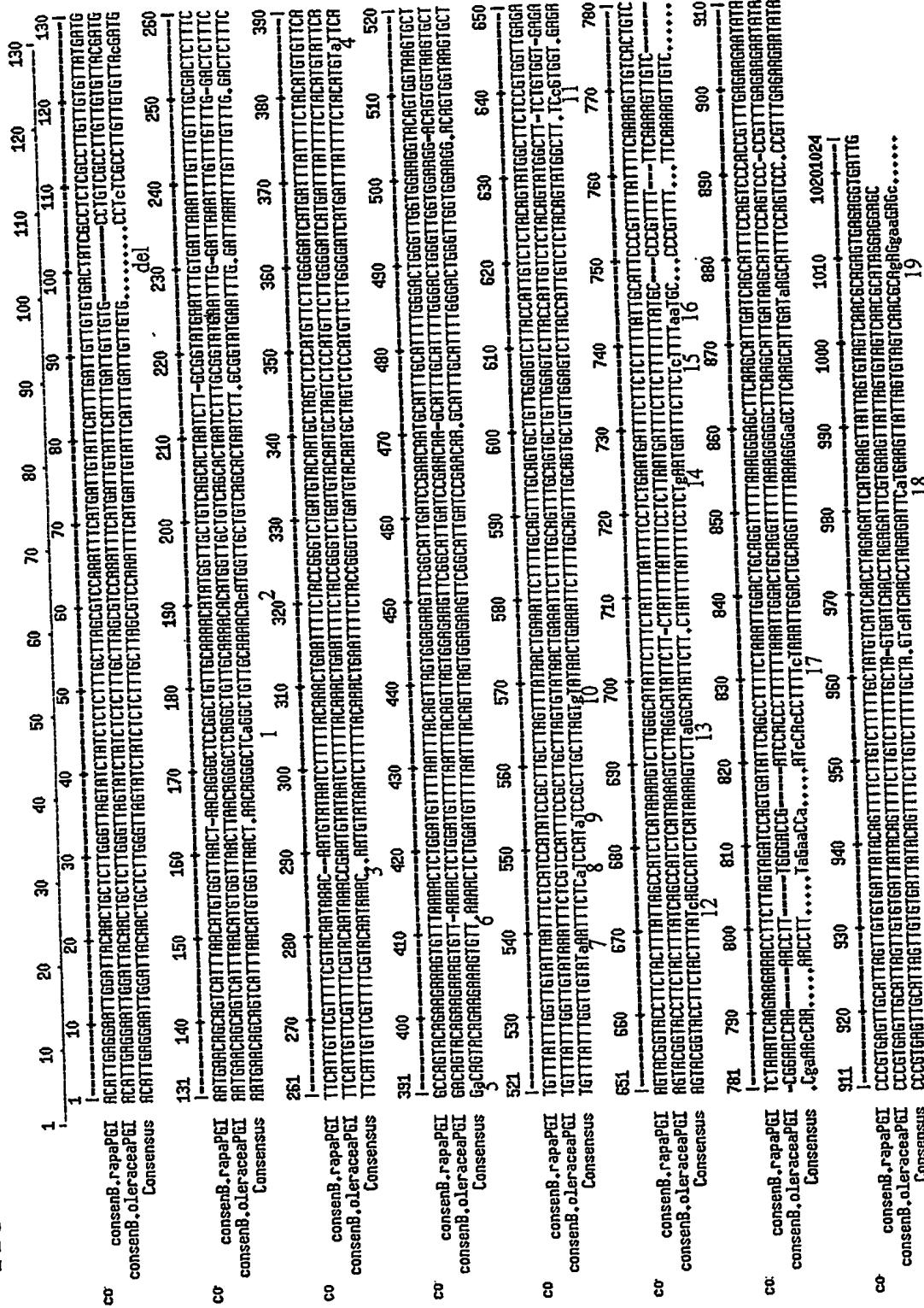


FIG 12



* one deletion and 19 substitutions, (n°2 is only in *B.rapa*)

Fig 13

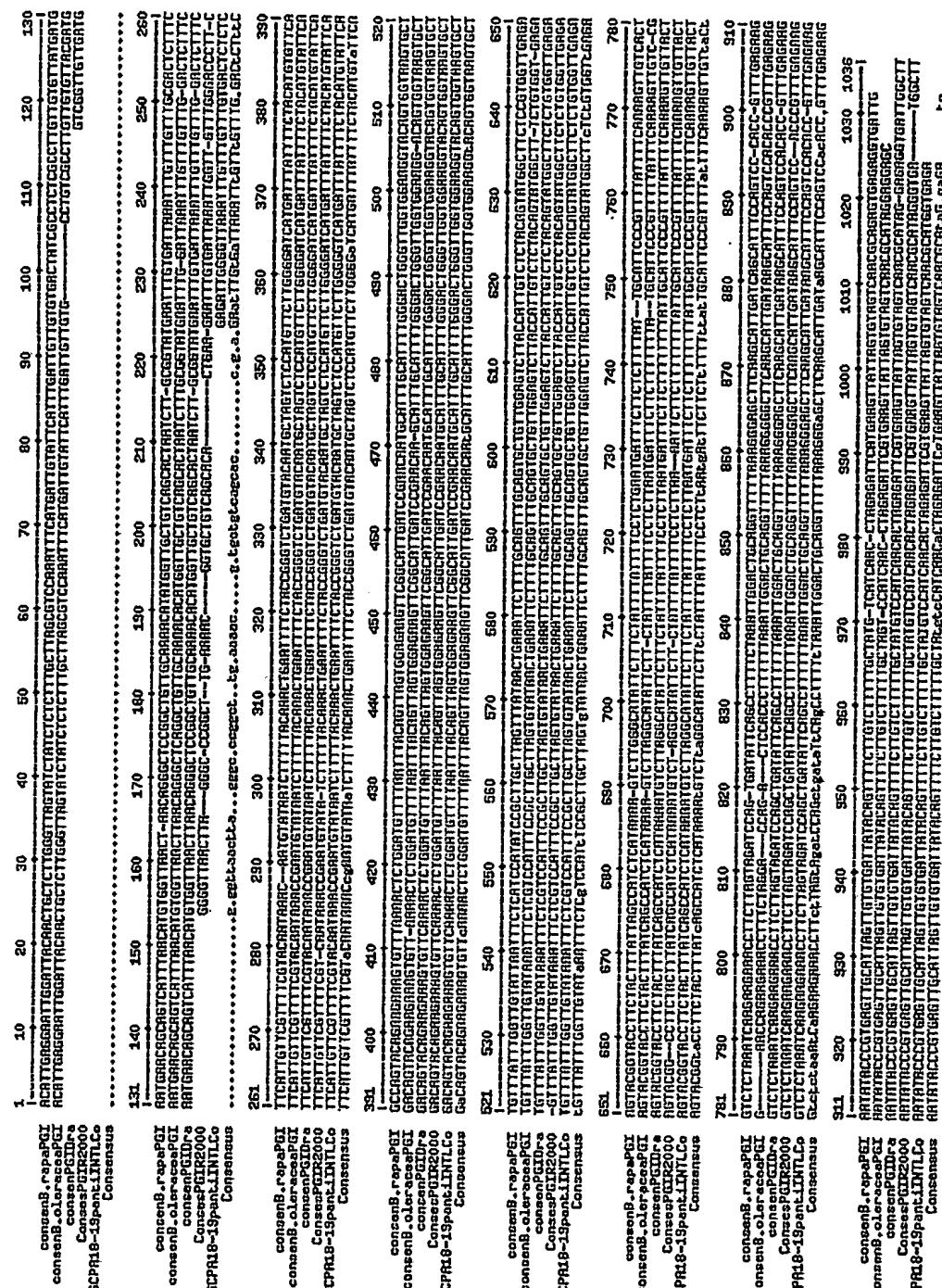
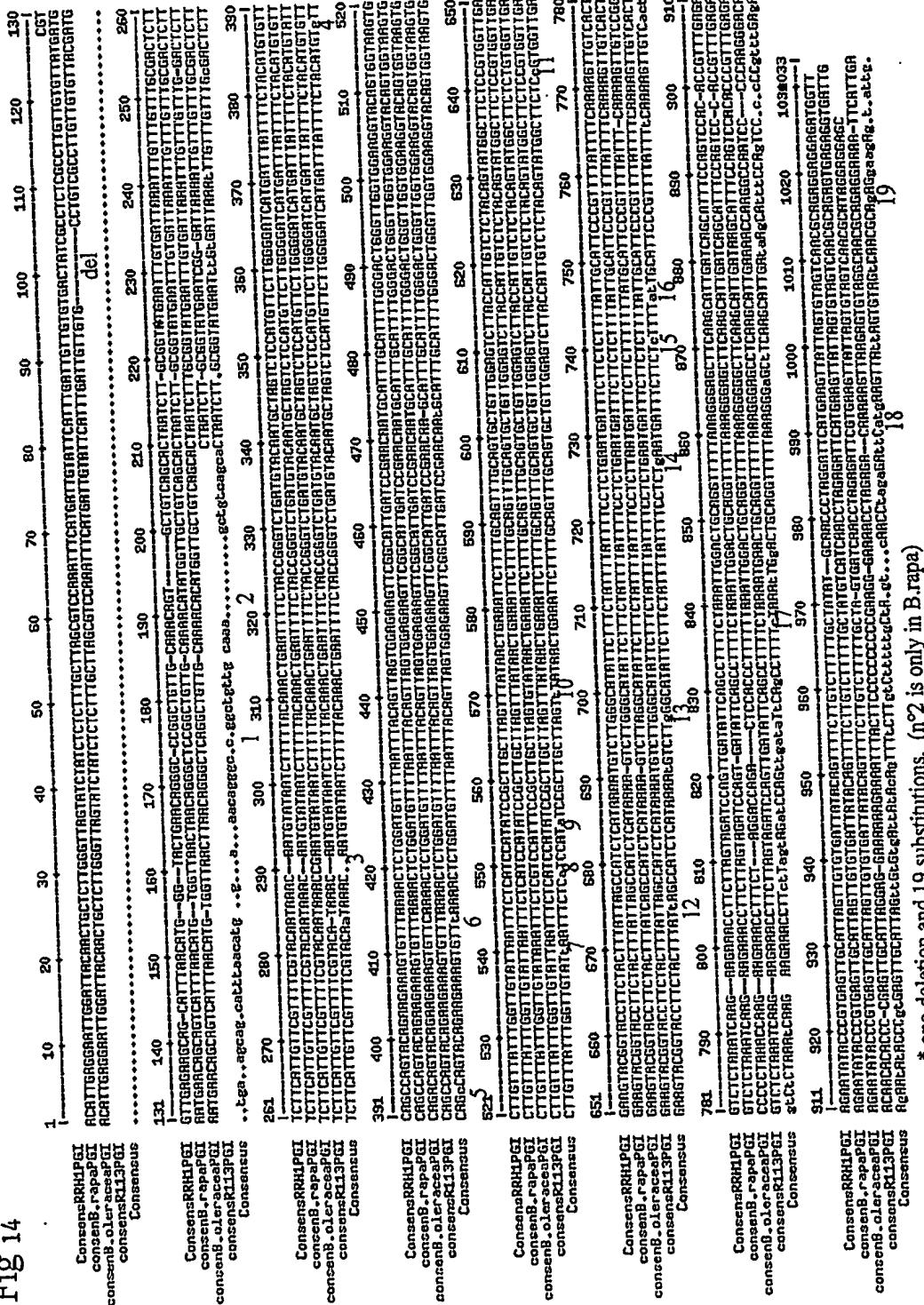


Fig 14



* One deletion and 19 substitutions, (11-2 is Only in Br. affs)

Fig 15

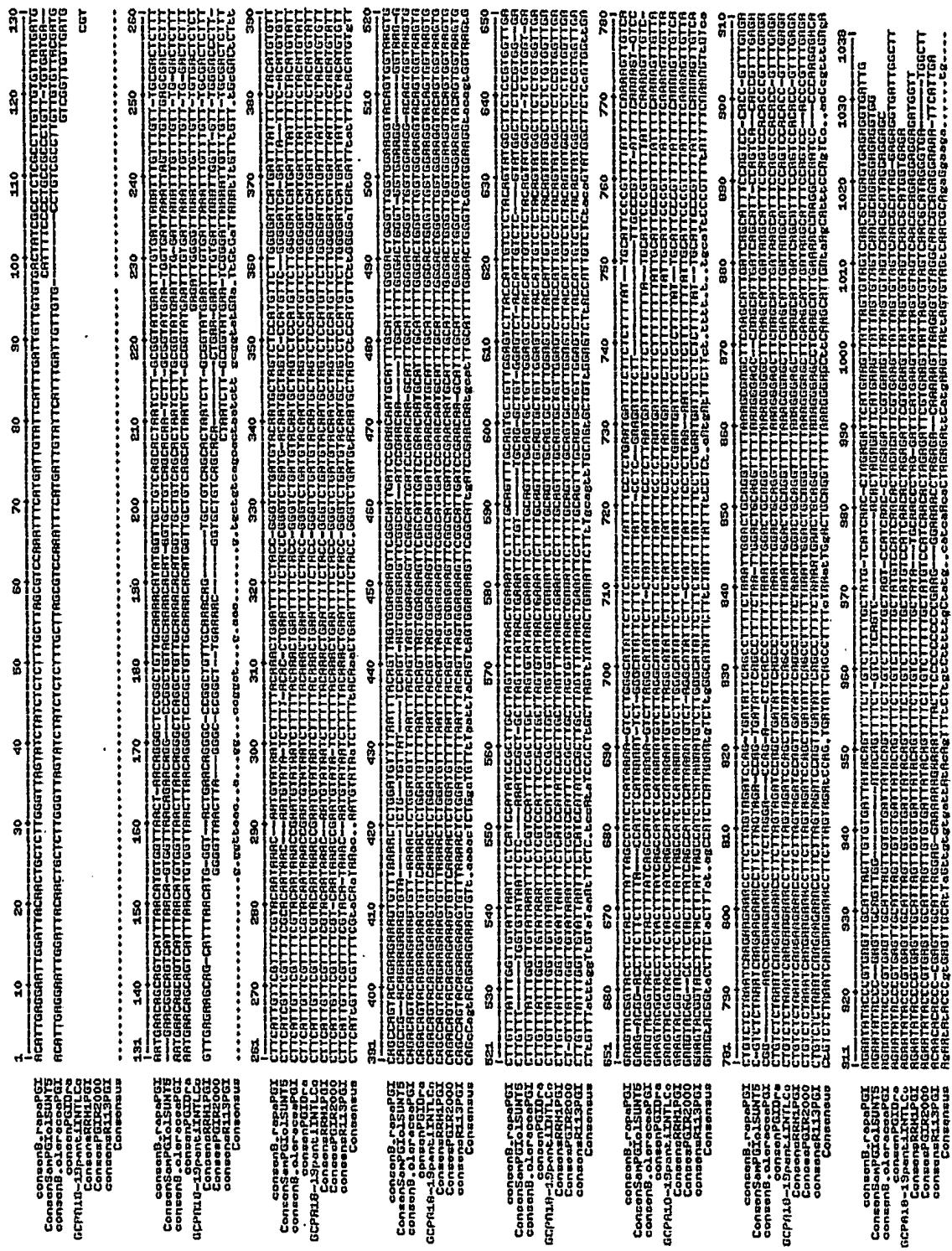


Fig. 16

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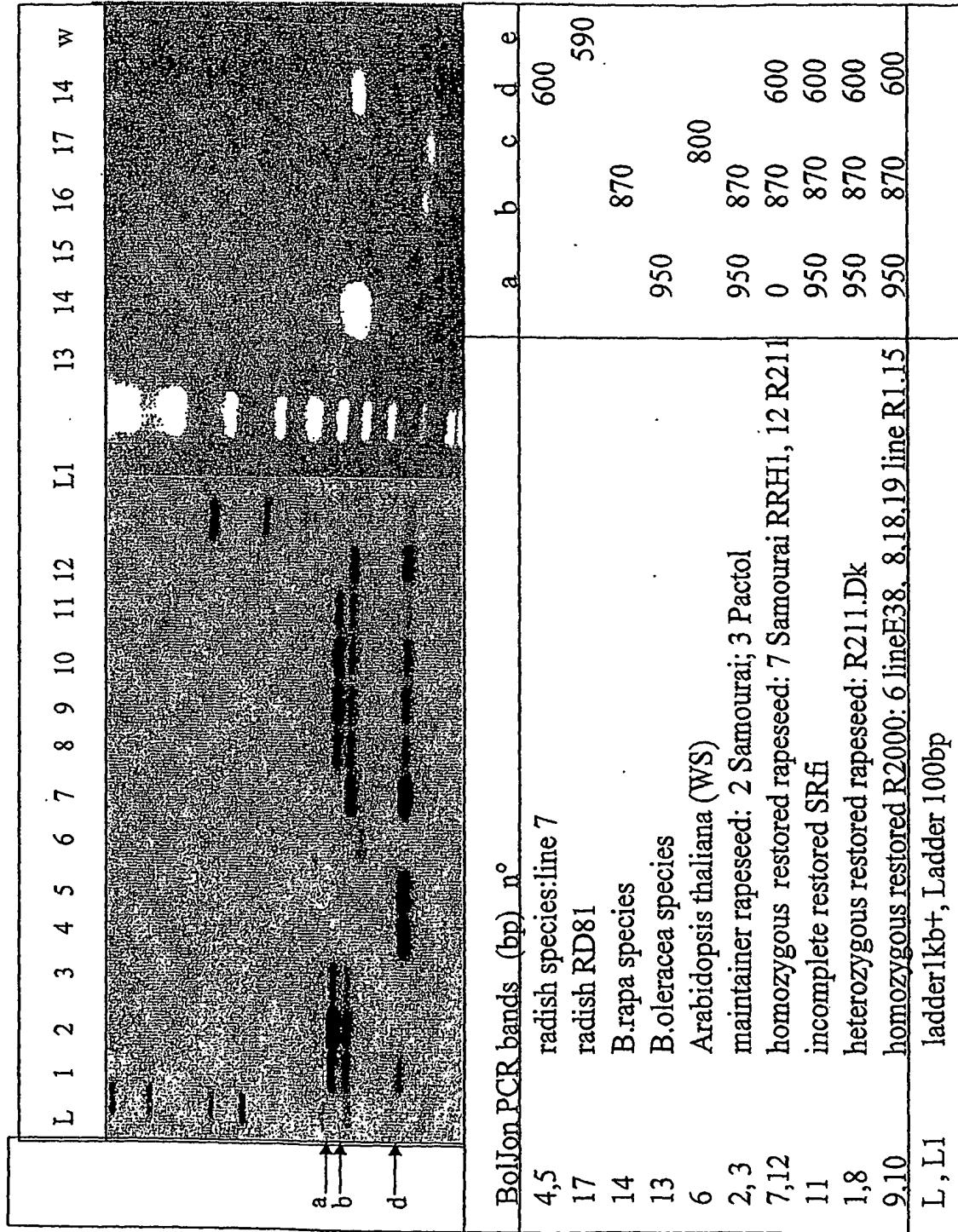


Fig 17

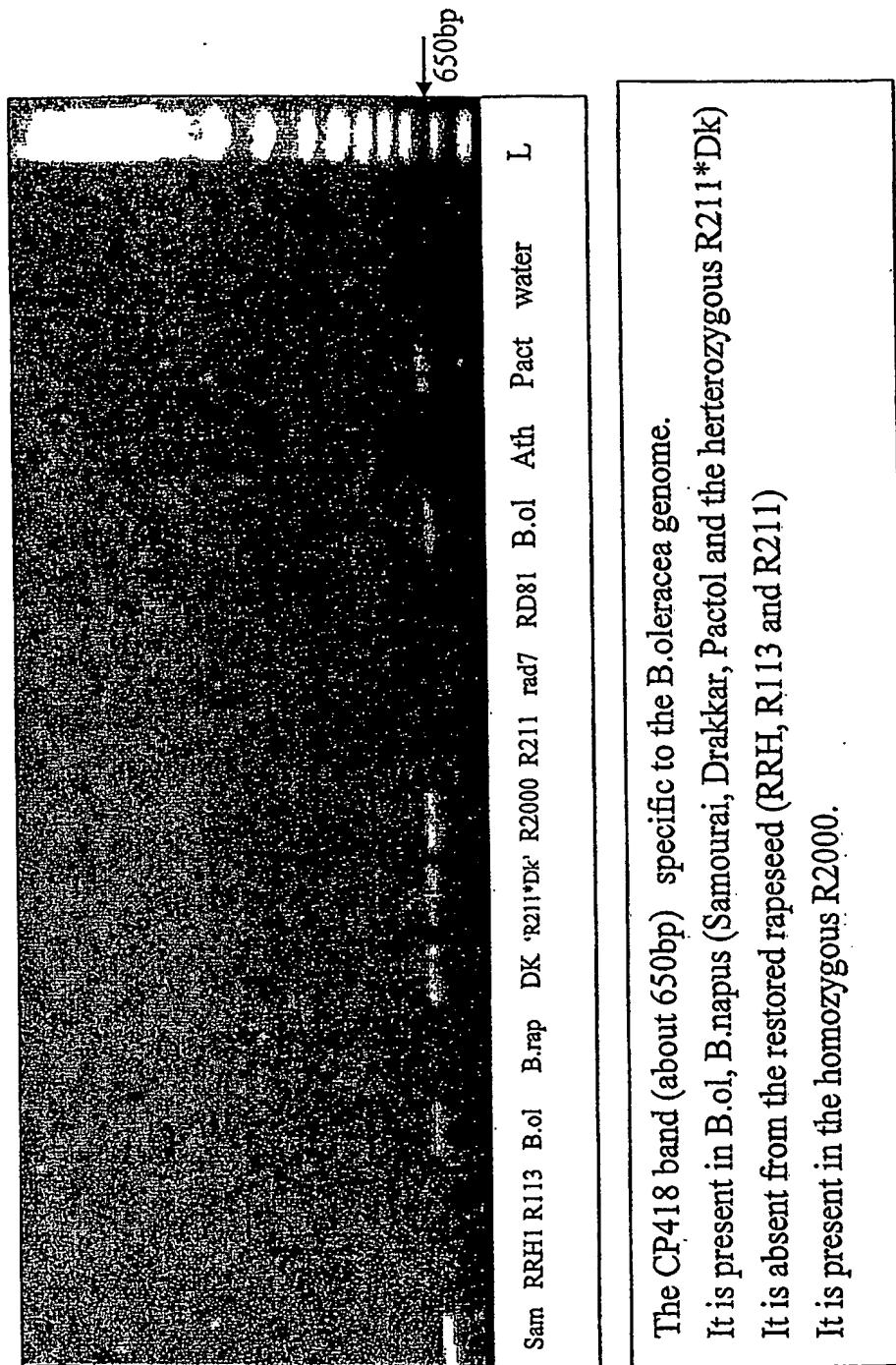


Fig 18

Marker	size bp	N°WRadrad RD81rad line7	Sam s	RRH1	R113	R211	R2000	R211*Dk	Draakkar Westroo	B.oleracea	B.rapa	Arabid.
PGIol	278	+	-	+	+	-	+	+	+	+	+	-
type B. ol	278					-	+	+	+	+	+	-
type B.rap	278			+	+	-						+
PGIUNT	950	+	-	+	+	-	+	+	+	+	+	-
type B. ol	950					-	+	+	+	+	+	-
type B.rap	950			+	+	-						+
PGI int												
radis	950	1000	+	-	+	-	-	-	-	-	-	-
Brassica	850	+	-	+	+	-	+	+	+	+	+	-
type B. ol	850					-	+	+	+	+	+	-
type B.rap	850			+	+	-						+
BolJön												
B. ole	950			+	-	-	+	+	+	+	+	-
B. rap	870			+	+	+	+	+	+	+	+	+
Ath	800											
radis	600			+	-	+	+	+	+	+	-	-
radis	590			+								

Brassicas and characterised by the DNA sequence

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